Purification, Characterization, and Sequencing of an Extracellular Cold-Active Aminopeptidase Produced by Marine Psychrophile *Colwellia psychrerythraea* Strain 34H

Adrienne L. Huston,1,* Barbara Methe,2 and Jody W. Deming1

University of Washington School of Oceanography, Seattle, Washington 98195,1 and The Institute for Genomic Research, Rockville, Maryland 208502

Received 9 October 2003/Accepted 26 February 2004

The limited database on cold-active extracellular proteases from marine bacteria was expanded by successful purification and initial biochemical and structural characterization of a family M1 aminopeptidase (designated ColAP) produced by the marine psychrophile *Colwellia psychrerythraea* strain 34H. The 71-kDa enzyme displayed a low optimum temperature (19°C) and narrow pH range (pH 6 to 8.5) for activity and greater thermolability than other extracellular proteases. Sequencing of the gene encoding ColAP revealed a predicted amino acid sequence with the highest levels of identity (45 to 55%) to M1 aminopeptidases from mesophilic members of the γ subclass of the *Proteobacteria* and the next highest levels of identity (35 to 36%) to leukotriene A4 hydrolases from mammalian sources. Compared to mesophilic homologs, ColAP had structural differences thought to increase the flexibility for activity in the cold; for example, it had fewer proline residues, fewer ion pairs, and a lower hydrophobic residue content. In addition to intrinsic properties that determine enzyme activity and stability, we also investigated effects of extracellular polymeric substances (EPS) from spent culture medium of strain 34H on ColAP activity at an environmentally relevant temperature (0°C) and at 45°C (the maximum temperature for activity). In both cases, ColAP stability increased significantly in the presence of EPS, indicating the importance of considering environmentally relevant extrinsic factors when enzyme structure and function are investigated.

Perennially cold environments comprise a significant portion of Earth’s biosphere (~80%), due largely to the presence of a predominantly cold ocean (<5°C) (2). The vast numbers of cold-adapted microorganisms which successfully inhabit these regions, which are referred to as psychrophilic organisms (optimal growth temperature \[T_{\text{opt}}\], ≤15°C; maximum growth temperature, ≤20°C) (45) and psychrotolerant organisms (organisms able to grow at low temperatures, but with a \[T_{\text{opt}}\] range of 20 to 35°C) (8), play vital roles in global elemental cycles and in the mineralization of organic matter. To do so, they must possess enzymes with sufficient activity to catalyze chemical reactions at low in situ temperatures.

Despite the environmental and physiological significance of these microorganisms, the mechanisms that allow enzymatic activity in the cold are currently not well understood. Low temperatures lead to exponential decreases in chemical rates, as described by the Arrhenius equation, and also tend to increase the compactness of proteins, thus limiting the conformational breathing necessary for catalysis (49). Despite these challenges, cold-active enzymes have evolved. Compared to mesophilic enzymes, these enzymes display three general distinguishing characteristics: a higher specific activity \([k_{\text{cat}}]\) or a catalytic efficiency \([k_{\text{cat}}/K_{m}]\) at temperatures between 0 and 30°C; a lower \[T_{\text{opt}}\] for activity; and limited stability in the presence of thermal increases and denaturing agents (24).

In this paper, we describe purification and initial biochemical and structural characterization of a cold-active, extracellular, family M1 aminopeptidase (designated ColAP) produced by the marine psychrophilic bacterium *Colwellia psychrerythraea* strain 34H. All cultured members of the genus *Colwellia* are cold adapted if they are not psychrophilic (7, 8). Representatives have been isolated from Arctic and Antarctic seawater and sea ice (6, 7, 38), as well as from cold deep-sea environments (20). Because the majority of organic matter in marine environments is composed of high-molecular-weight compounds that are largely unavailable for direct uptake by heterotrophic bacteria, the hydrolytic activity of extracellular enzymes plays a crucial role in bacterial acquisition of dissolved organic matter (as described by Vetter et al. [58]). The role of this activity is thought to be particularly important in low-temperature environments, in which bacterial activity is believed to require higher levels of dissolved organic matter than the bacterial activity in warmer environments requires (48). Of the classes of extracellular enzymes that have been examined, proteolytic activity has consistently been found to be the dominant activity throughout the marine environment (14, 34, 53), indicating its importance in the transformation and bacterial acquisition of nitrogen-rich organic compounds. However, few extracellular proteases from marine organisms have been characterized in order to better understand the mechanisms that enable their activity in situ (21). Aside from their environmental and ecological importance, cold-active proteolytic enzymes are also useful for investigations of the structural basis of protein stability and offer great potential for biotechnological applications (10, 27). We compared the activity and structural characteristics of the newly purified enzyme ColAP to the activities and structural characteristics of homologous enzymes from mesophilic organisms in an attempt to clarify features unique to cold activity. We also examined the po
tial stabilizing effect on ColAP of extracellular polymeric sub-
stances (EPS) extracted from spent culture medium of strain
34H in order to begin to address the importance of extrinsic
factors in facilitating the activity of cold-active extracellular
enzymes in the environment.

MATERIALS AND METHODS

Bacterial strain, culture conditions, and crude enzyme concentration.
The marine psychrophilic proteobacterium strain 34H belonged to the γ subclass of the
Proteobacteria (γ-proteobacteria) was isolated from surficial sediments of the
continental shelf off northeast Greenland (79°43′N, 161°4′W) from a depth of
305 m (33). The strain was purified by the dilution-to-extinction approach by
using marine broth 2216 (Difco Laboratories), the same medium used for rou-
tine culturing in this study. The empirically observed temperature growth range
of this organism is −6 to 19 °C, and the T_{opt} for growth is 8 to 9 °C (35); the
highest levels of proteolytic activity in the culture medium are observed at low
growth temperatures (−1 to 2°C) during the late log to early stationary phase.
For protease purification studies, a logarithmic-phase culture was inoculated
into 20 liters of marine broth 2216 at 2°C in aerated (bubbled air passed through
a 0.2-μm- pore-size filter) and stirred 25-liter carboys. Cells were harvested at the late
log phase as determined by measuring the optical density at 600 nm. To ob-
tain the extracellular fraction, the culture was centrifuged at 3,400 × g for 20 min
at 4°C and then filtered through a 0.45-μm-pore-size Gelman membrane capsule.
The extracellular extract was concentrated approximately 25-fold above a 10,000-
Da spiral-wound membrane cartridge by using a tangential-flow ultrafiltration unit.
The filtration and concentration steps were performed at 2°C. The concentrated
extract was amended with 10% glycerol and frozen at −80 °C for future studies.

Enzyme assays. Proteolytic activity in the crude extracellular extract was
assayed by using the fluorescently tagged substrate analog 1-leucine-7-amido-
4-methylcoumarin (MCA-L) (Sigma) to measure leucine aminopeptidase
(LAPase) activity in an artificial seawater (ASW) buffer (0.4 M NaCl, 9 mM KCl,
26 mM MgCl2, 28 mM MgSO4, 5 mM TAPSO [N-Tris(hydroxymethyl)methyl]-3-
amino-2-hydroxypropanesulfonic acid]) (pH 7.2). Briefly, 25 to 250 μl of a
suitable dilution of enzyme solution was added to 2.5 ml of ASW buffer. Sub-
strate was added at a saturating concentration (200 μM), as determined by
substrate saturation experiments. Activity was measured in duplicate samples
during time course experiments with a Perkin-Elmer LS-5B spectrophotometer
at excitation and emission wavelengths of 355 and 440 nm, respectively.

During purification, activity was assayed routinely with MCA-L in ASW buffer.
LAPase activity was also measured by using 200 μM Ala-Ala-Pro-Leu p-nitro-
anilide (AAPL-p-nitroanilide) as the substrate in ASW buffer during time course
experiments with a spectrophotometer at 410 nm with an extinction coeffi-
cient of 8,480 M⁻¹ cm⁻¹, as described above. General proteolytic activity was assayed by
using the macromolecular substrate azocasein in a reaction mixture containing
300 μg azocasein (wt/vol) in ASW buffer. The reaction was terminated after several hours by adding 500 μl of
1% trichloroacetic acid. The activity of the supernatant
substrate was determined by the method of Bradford by
electrophoresis. Fractions from the various fast protein liquid chromatogra-
fy purification steps were subjected to sodium dodecyl sulfate-polyacrylamide
gel electrophoresis (SDS-PAGE) analysis by using denaturing 12% polyacry-
lamide gels. SYPRO Ruby protein gel stain (Molecular Probes) was used for
protein visualization. The method used for SDS-PAGE was essentially the
method described by Laemmli (41).

Extraction of EPS. C. psychrerythraea strain 34H was grown at 2°C and
harvested at the late log phase, as determined by optical density at 600 nm, to
obtain a crude EPS extract. Extraction was performed as previously described (3, 19), with the following modifications. Cells were removed from the late-log-phase
culture by gentle centrifugation at 1,500 × g for 50 min at 2°C and subsequent
filtration of the supernatant through a GF/F filter. Three volumes of chilled 100%
ethanol was added to the filtered supernatant, which was then incubated over-
night at 2°C. EPS formed a precipitate that was collected by centrifugation
at 10,000 × g for 20 min. The ethanol wash and centrifugation steps were repeated
three times. To remove low-molecular-weight polysaccharides, the final pellet
was dissolved in distilled water and dialyzed for 48 h at 2°C by using Spectra Por
dialysis tubing (2,000- to 3,500-Da cutoff). The resulting dialysate was frozen at −20°C
for future use. EPS was quantified by the colorimetric phenol-sulfuric acid method (22).

Enzyme characterization. Michaelis-Menten kinetic parameters for the activity
of the purified enzyme, which was later determined to be a member of the MI
family of aminopeptidases and was designated ColAP, were determined from
substrate saturation assays at −1, 9, and 19°C in ASW buffer by using MCA-L at
various concentrations. Values were corrected to 20°C with the following modi-
fication to the temperature range and T_{opt} for activity of the enzyme, 0.1 μg
of purified ColAP was incubated in duplicate at −1, 4, 7.5, 12, 16, 19, 26, 28, 35,
and 46°C for 2 h in ASW buffer with 200 μM MCA-L as the substrate. Thermal
stability was determined by incubating 0.1 μg of ColAP in duplicate at 30, 40, 45,
and 50°C for up to 45 min, as well as at 0°C for 45 h, in ASW buffer. At 0 and
45°C, ColAP was also incubated with an environmentally relevant concentration
of EPS (33 μg of C m⁻³) (39). Fractions incubated at the higher temperatures
(30 to 50°C) were first placed on ice to stop the inactivation reaction; the residual
activity in each case was then measured after equilibration of the sample at 20°C.
To determine the effect of pH on activity, 0.1 μg of ColAP was assayed in
duplicate at 20°C at pH values ranging from 5.0 to 9.0 in buffer-amended ASW
by using 200 μM MCA-L as the substrate. The following buffers were used at a
concentration of 20 mM: acetic acid (pH 5.0); morpholinenuethanesulfonic acid (MES)
(pH 6.0); PIPES (pH 6.5 and 7.0); TRIZMA [2-amino-2-(hydroxymeth-
yl)-1,3-propanediol] (pH 7.5, 8.0, and 8.5); and AMPSO [N,N-dimethyl-2-hy-
droxyethyl]-3-amino-2-hydroxypropanesulfonic acid] (pH 9.0).

For inhibition and metal studies, ColAP was incubated with different inhibitors
(HED, EDTA-2Na, dithiothreitol, and divalent ions (CaCl2,
MgCl2, MnCl2, and ZnCl2) for 1 h on ice in 20 mM Tris buffer containing 0.4 M
NaCl (pH 7.2). Residual activity was measured at 20°C by using MCA-L as the
FIG. 1. Superose 12 gel filtration chromatography profile of the proteolytic activity in a crude extracellular extract from a late-log-phase culture of C. psychrerythraea strain 34H at 2°C. A 500-µl sample of concentrated extract (see text) was gravity loaded onto a column which was preequilibrated with 10 mM Tris–0.3 M NaCl (pH 7.3), eluted with the same buffer at a rate of 1.0 ml min⁻¹, and collected in 1-ml fractions. Fractions were assayed for proteolytic activity as described in Materials and Methods by using the following substrates: MCA-L (■), AAPL-p-nitroanilide (□), and azocasein (●). The arrows indicate the positions of molecular masses (in kilodaltons).

N-terminal amino acid sequencing. The N-terminal sequence of the purified enzyme was obtained by electrophotoblotting SDS–12% PAGE gels loaded with ColAP onto polyvinylidene difluoride membranes, which were subsequently stained with Coomassie blue R-250. An automated Edman-type (23) analysis was performed with excised protein bands by using a Perkin-Elmer Applied Biosystems model 494 Procise protein sequencer with an online 140C PTH amino acid analyzer. Electrophotoblotting and protein sequencing were performed by the Iowa State University Protein Facility.

TABLE 1. Purification of ColAP, a cold-active M1 aminopeptidase from C. psychrerythraea strain 34H

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)a</th>
<th>Sp act (U mg⁻¹)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>6.2</td>
<td>3.3</td>
<td>0.54</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Sepharose Q</td>
<td>0.72</td>
<td>2.1</td>
<td>2.9</td>
<td>5.4</td>
<td>64</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>0.0054</td>
<td>1.0</td>
<td>185</td>
<td>340</td>
<td>30</td>
</tr>
<tr>
<td>Resource Q</td>
<td>0.0032</td>
<td>0.80</td>
<td>250</td>
<td>460</td>
<td>24</td>
</tr>
</tbody>
</table>

a One unit was defined as degradation of 1 nmol of MCA-L min⁻¹.

RESULTS

Production of extracellular proteases by strain 34H. The results of gel filtration chromatography of concentrated extracellular extracts indicated that C. psychrerythraea strain 34H produces several extracellular proteolytic enzymes with different specificities (Fig. 1). Three peaks of azocasein digestion were observed in column eluant fractions corresponding to molecular masses of 71 to 83, ~43, and ~22 kDa. Additional peaks of MCA-L and AAPL-p-nitroanilide hydrolysis were also observed; in each case they corresponded to a molecular mass of 71 to 83 kDa.

Purification of ColAP. ColAP was purified to homogeneity (Fig. 2), as summarized in Table 1. Purification was difficult, even after we paid attention to the temperature, due to the sensitivity of the enzyme to standard purification procedures.
such as variations in the pH and the salt concentration. Ion-exchange and hydroxyapatite columns resulted in greater retention of ColAP activity than the other techniques tested (e.g., hydrophobic interaction chromatography). Hydroxyapatite techniques resulted in significant purification (~340-fold increase in specific activity), while the final ion-exchange step consistently removed a 50-kDa contaminant (barely visible in Fig. 2). The purification procedure resulted in a relatively high yield (24%) with a ~460-fold increase in specific activity. The molecular mass of purified ColAP was ~71 kDa as determined by both SDS-PAGE and gel filtration analysis, indicating that ColAP is a monomeric enzyme.

Kinetic parameters and thermodependence of activity. The highest specific activity (k_cat) for ColAP (0.36 s^{-1}) was observed at ~19°C (Table 2). The enzyme showed no significant activity at 46°C, but 12% of the maximal activity was still observed at the lowest temperature tested, ~1°C (Fig. 3). The half-saturation coefficient (K_m) for ColAP was lowest at ~1°C (43 μM) and increased with increasing temperature (to 72 μM at 19°C) (Table 2). The highest k_cat/K_m value for ColAP (5.0 s^{-1} mM^{-1}) was also observed at 19°C, and 44% of this value retained at 9°C (the T_{opt} for growth of strain 34H); 20% of the value was retained at ~1°C, an environmentally relevant temperature (Table 2). The activation energy for cold-active ColAP was 71 ± 1.5 kJ mol^{-1} (Table 2).

Thermostability of activity. ColAP exhibited great sensitivity to heat, and the calculated half-lives were ~67, 38, 10, and 5 min at 30, 40, 45, and 50°C, respectively (Fig. 4A). In the presence of EPS extracted from spent culture medium, the stability of ColAP increased by a factor of 17 at 45°C, and the observed half-life was 170 min (Fig. 4A). At 0°C (Fig. 4B), the half-life of ColAP in ASW buffer alone was about 18 h, while no detectable loss of activity was observed in the presence of EPS after incubation for 45 h. Furthermore, at both 45 and 0°C, the initial activity appeared to be higher (by a factor of 1.3 to 2.7) in the EPS-amended fractions than in the ASW controls. No ColAP activity was detected in the EPS extract itself.

Effect of pH on activity. ColAP exhibited a narrow pH range for activity, and the maximal activity was observed at neutral pH. At pH 6.0 and 8.5, only 10 and 5% of the maximal activity was retained, respectively, while no activity was detected at pH 5.0 or 9.0. No recovery of activity was observed when the preparation was returned to neutral pH.

Effects of inhibitors and ions on enzyme activity. Of the potential inhibitors tested (Table 3), phenylmethylsulfonyl fluoride (a serine protease inhibitor) did not affect enzyme activity significantly, while 10 mM dithiothreitol (a reducing agent) and all concentrations of EDTA (a metal-chelating agent)

---

**TABLE 2. Kinetic parameters for purified ColAP**

<table>
<thead>
<tr>
<th>Assay temp (°C)</th>
<th>k_cat (s^{-1})</th>
<th>K_m (μM)</th>
<th>k_cat/K_m (s^{-1} mM^{-1})</th>
<th>E_a (kJ mol^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1</td>
<td>0.043 ± 0.006</td>
<td>43 ± 22</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.13 ± 0.02</td>
<td>58 ± 31</td>
<td>2.2</td>
<td>71 ± 1.5</td>
</tr>
<tr>
<td>19</td>
<td>0.36 ± 0.06</td>
<td>72 ± 34</td>
<td>5.0</td>
<td></td>
</tr>
</tbody>
</table>

*E_a* was calculated from an Arrhenius analysis of the k_cat data.
tested completely inhibited ColAP activity. Of the divalent cations tested, Zn$^{2+}$ and Mn$^{2+}$ strongly inhibited activity (Table 3), while Ca$^{2+}$ was slightly inhibitory. Mg$^{2+}$ stimulated ColAP activity at a concentration of 10 mM or higher (equivalent to the concentration found in seawater), as shown by the value for ASW buffer in Table 3. Activity also appeared to be strongly dependent upon chloride salts; no activity was seen in 20 mM Tris buffer lacking NaCl (Table 3).

**Substrate preference.** Purified ColAP displayed the highest levels of activity with methylcoumarin substrates containing alanine and arginine residues and intermediate levels of activity with leucine (Table 4). Lower levels of activity were observed with serine, while no detectable activity was observed with aspartic acid and proline residues and intermediate levels of activity with methylcoumarin substrates containing glycine. Activity was also observed with AALP p-nitroanilide and the macromolecular substrate azocasein (Table 4).

**Sequence analysis and protein modeling.** The predicted amino acid sequence of ColAP (35) was examined to determine its similarity to sequences available in the National Center for Biotechnology Information database. ColAP exhibited the highest overall levels of amino acid identity (45 to 55%) with putative M1 aminopeptidases from mesophilic members of the same group of bacteria ($\gamma$-proteobacteria) and the next highest levels of identity (35 to 36%) with LTA$_4$ hydrolases from various sources (Fig. 5). The results of a multiple-sequence alignment indicated that there was perfect conservation of the putative substrate binding site (GGMEN), the zinc binding motif (HEXXH-X$_{18}$-E), and catalytic residues involved in aminopeptidase activity (Glu 301 and Tyr 386 in donors, respectively [4, 54, 59]).

Bicompartmental sequence analysis revealed amino acid identities distributed throughout the entire ColAP sequence that corresponded to secondary structural elements. As a result, the structural model of ColAP (35) had three domains that together created a cleft containing a catalytic zinc site similar to that of LTA$_4$ hydrolase (54).

In our analysis of the sequences and models of ColAP and its mesophilic equivalents (Table 5) we focused on structural features believed to increase the structural flexibility usually associated with low-temperature enzyme activity. In ColAP the number of proline residues, which affect the backbone flexibility and thus the local mobility of the chain, is less than the numbers of proline residues in the other enzymes. The number of arginine residues, which have the potential to form multiple ion pairs and H bonds, is less than the number of lysine resi-

### Table 3. Effects of different compounds on ColAP activity

| Buffer or compound | Concentration (mM) | ColAP activity (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control buffer</td>
<td>100 ± 7</td>
<td></td>
</tr>
<tr>
<td>Inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride</td>
<td>0.1</td>
<td>91 ± 20</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>1</td>
<td>75 ± 1</td>
</tr>
<tr>
<td>EDTA</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Divalent cations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZnCl$_2$</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>1</td>
<td>84 ± 5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>1</td>
<td>100 ± 15</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>122 ± 10</td>
</tr>
<tr>
<td>MnCl$_2$</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Control buffer without NaCl</td>
<td>189 ± 15</td>
<td></td>
</tr>
<tr>
<td>ASW buffer</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Purified ColAP (0.1 μg) was incubated with various compounds for 1 h on ice in control buffer (20 mM Tris, 0.4 M NaCl; pH 7.2); residual activity was measured at 20°C.*

### Table 4. Relative activities of ColAP with various substrates at pH 7.2 and 20°C

| Substrate | Hydrolysis rate (μmol min$^{-1}$ mg$^{-1}$) | Relative activity (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Leucine 7-amido-4-methylcoumarin</td>
<td>0.33 ± 0.030</td>
<td>100</td>
</tr>
<tr>
<td>L-Alanine 7-amido-4-methylcoumarin</td>
<td>1.2 ± 0.080</td>
<td>364</td>
</tr>
<tr>
<td>L-Arginine 7-amido-4-methylcoumarin</td>
<td>1.1 ± 0.10</td>
<td>333</td>
</tr>
<tr>
<td>L-Proline 7-amido-4-methylcoumarin</td>
<td>0.92 ± 0.015</td>
<td>0</td>
</tr>
<tr>
<td>L-Serine 7-amido-4-methylcoumarin</td>
<td>0.040 ± 0.030</td>
<td>6.1</td>
</tr>
<tr>
<td>AAPL p-nitroanilide</td>
<td>0.13 ± 0.030</td>
<td></td>
</tr>
</tbody>
</table>

*—, no detectable activity.*
dyes, as shown by the decrease in the Arg/(Arg + Lys) ratio. ColAP is also characterized by having a lower content of hydrophobic residues (A, F, I, L, M, P, V, and W) and a higher content of charged residues (D, E, H, K, and R) than the other enzymes. Parameters resulting from bioinformatics of the complete amino acid sequences of ColAP and its mesophilic homologs with the ProtParam tool indicated that the theoretical pI and grand average of hydropathicity (40) of ColAP are lower (5.25 versus 5.28 to 6.49 and 0.343 versus 0.350, respectively), suggesting that the hydro solubility of ColAP is higher and that this enzyme has a better interaction with the solvent. The results of 3D modeling also support these findings: the solvent-accessible surface of ColAP has a lower number of hydrophobic residues and a higher number of charged residues than the surfaces of its counterparts from mesophilic \( \gamma \)-proteobacteria. Modeling analysis further suggested that ColAP has fewer ion pairs (15 versus 17 to 21), which leads to a decrease in the number of intrinsic stabilizing bonds. The enzymes did not differ in other indices thought to confer low-temperature activity, such as the aliphatic index (36) and aromatic interactions (data not shown).

**DISCUSSION**

In this study, we developed a successful protein purification scheme that allowed us to investigate the cold activity of an extracellular aminopeptidase (ColAP) produced by a marine psychrophile that represents a bacterial genus that is known to occur in a variety of cold marine environments. C. psychrerythraea strain 34H is particularly useful as a model organism for cold adaptation studies because the sequence of its whole genome has suggested that strain 34H is capable of producing many more extracellular proteolytic enzymes than the enzymes observed in this study; at least 90 open reading frames have been classified as having roles related to protein and peptide degradation, and 51 of the proteins encoded by these open reading frames contain predicted signal peptides which suggest transport from the cytoplasm and a possible role in extracellular protein degradation (35).

Inhibition studies with EDTA confirmed that the ColAP enzyme that we purified from the exudates of strain 34H is a metalloprotease, while sequence analysis suggested that zinc is required for activity of this enzyme. Zinc, however, inhibited ColAP activity at the concentrations tested. Although the mechanism is currently unknown, similar results have been obtained for other zinc-dependent aminopeptidases, such as thermolysin and LTA4 hydrolase, when they have been incubated with zinc at a concentration greater than that required for activity (molar ratios greater than 1) (32, 60). In contrast, other salts common in seawater either stimulated ColAP activity (magnesium) or appeared to be required for activity (chloride). More detailed studies of chloride effects on ColAP may reveal anion binding site, given other relationships between ColAP and LTA4 hydrolase, an enzyme in which chloride stimulation obeys saturation kinetics (29).

ColAP also resembles LTA4 hydrolase in terms of substrate preference; both enzymes appear to prefer arginine and alanine substrates (29). The observed hydrolytic activity against AAPL \( p \)-nitroanilide and azocasein suggests that ColAP has both endopeptidase and exopeptidase activities. The fact that the specific activities of LTA4 hydrolase with synthetic substrates, such as \( p \)-nitroanilide and \( \beta \)-naphthylamide derivatives of amino acids, are significantly lower than the specific activities with dipeptides and tripeptides (28) may help explain the observed low specific activity of ColAP with synthetic methylecumin substrates. Further work is needed to determine the specific activities of ColAP with dipeptide and tripeptide substrates.

Activity characterization revealed that the \( T_{opt} \) for ColAP activity (19°C) is low relative to the \( T_{opt} \)s of previously described extracellular proteases (whether they were purified from mesophilic, psychrotolerant, or psychrophilic bacteria), which generally have been found to exhibit maximal activity at temperatures between 30 and 60°C (10, 21). In agreement with the current view that low-temperature enzymatic activity is associated with reduced structural stability, ColAP exhibited a narrow pH range (pH 6.0 to 8.5) for activity compared to the pH ranges of previously characterized cold-active proteases (pH 5 to 11) (46, 50). Furthermore, in the absence of stabilizing agents, ColAP displayed a thermolability equal to or greater than the thermostability of other extracellular proteases from psychrophilic and psychrotolerant sources (11, 42, 46).

However, the lifetime of a cell-free enzyme in a salt solution lacking other dissolved or particulate organic compounds does

### TABLE 5. Summary of structural parameters of ColAP, potentially involved in adaptation to low temperatures, and M1 aminopeptidases from mesophilic members of the \( \gamma \)-proteobacteria

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>C. psychrerythraea ColAP</td>
<td>25</td>
<td>0.35</td>
<td>43.8</td>
<td>32.8</td>
<td>23.4</td>
<td>5.25</td>
<td>−0.343</td>
<td>15</td>
<td>28.3</td>
</tr>
<tr>
<td>Mesophile M1 aminopeptidases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>32</td>
<td>0.56</td>
<td>45.2</td>
<td>35.8</td>
<td>19.0</td>
<td>5.75</td>
<td>−0.257</td>
<td>17</td>
<td>30.9</td>
</tr>
<tr>
<td>Shewanella oneidensis</td>
<td>43</td>
<td>0.42</td>
<td>47.6</td>
<td>29.7</td>
<td>22.2</td>
<td>5.28</td>
<td>−0.282</td>
<td>19</td>
<td>34.5</td>
</tr>
<tr>
<td>Xanthomonas campestris</td>
<td>44</td>
<td>0.40</td>
<td>47.9</td>
<td>29.9</td>
<td>22.2</td>
<td>5.46</td>
<td>−0.261</td>
<td>18</td>
<td>34.4</td>
</tr>
<tr>
<td>Xylella fastidiosa</td>
<td>34</td>
<td>0.43</td>
<td>46.6</td>
<td>31.0</td>
<td>22.4</td>
<td>6.49</td>
<td>−0.178</td>
<td>18</td>
<td>32.2</td>
</tr>
<tr>
<td>Mesophile MR-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthomonas axonopodis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* GRAVY, grand average of hydropathicity.
not have physiological or environmental relevance. Many microorganisms in natural environments live in assemblages that form organic-rich flocs and biofilms (15); the highest concentrations of cell-free enzymes in cold marine environments are usually found in such organic-rich sites, including detrital aggregates (34), sediments (57), and sea ice (30). These enzymes are likely to be embedded in organic matrices or to be associated with high-molecular-weight materials (such as EPS) found in phytodetritus and sea ice (39). Our observation that EPS has significant effects on the half-life of ColAP, especially at 0°C, suggests that EPS may have an important stabilizing role for ColAP in an environmental context. Although we did not characterize the components of our EPS extract or the enzyme-stabilizing mechanism, the results of previous studies suggest that EPS (as well as compatible solutes) act nonspecifically via preferential exclusion mechanisms that create an entropically unfavorable state, thus favoring the folded state over the unfolded state of proteins (55, 56). The hydrated property of EPS may also act as a buffer that maintains extracellular enzyme activity during local chemical and osmotic changes (18), while it also protects enzymes from proteolysis. Furthermore, marine polymer gels (such as EPS) have been found to sequester high levels of Mg²⁺ and Ca²⁺ relative to the levels in the surrounding seawater (12), which may further stabilize the structure and stimulate the activity of metalloenzymes such as ColAP.

Regardless of the molecular mechanisms, the longer ColAP lifetime at 0°C mediated by the presence of EPS easily matches or exceeds the observed generation times of microbial assemblages in samples of Arctic seawater (43) and sinking particles (35). The stabilizing effect of EPS may facilitate the selection of cold-active extracellular enzymes by enhancing their benefits to the producing (and other nearby) organisms over several microbial generations. By helping to maintain enzyme function, whether in low- or high-thermal-energy environments (26), extrinsic factors such as EPS may increase the range of extreme environments that bacteria can inhabit successfully.

At the intrinsic level, the current working hypothesis is that cold-active enzymes have increased flexibility in certain parts of their structures to accommodate the substrates and molecular movement necessary for catalysis at low temperatures, a flexibility that also results in reduced structural stability (17). As found in other studies (16, 25), the observation that all amino acids putatively involved in the reaction mechanism are strictly conserved in ColAP when this enzyme is compared to its mesophilic homologs suggests that molecular adaptation to cold lies elsewhere in the protein structure. The numerous structural differences observed when the sequence and 3D model of ColAP were compared to the sequences and 3D models of its homologs are consistent with increased protein flexibility and the observed low-temperature activity and reduced stability of ColAP. Whether such differences are indeed responsible for the enhanced structural flexibility that is thought to allow activity at low temperatures remains to be determined mechanistically. When subjected to statistical analysis, purported rules for amino acid substitution in different thermal classes of enzymes have yielded insignificant results (5, 47); this is not surprising, since the stabilization energies of mesophilic and extremeophilic enzymes differ by the equivalent of only a few noncovalent interactions (37). Also, the taxonomic differences among source organisms when enzymes from different thermal regimens are compared increase the likelihood of variable selective pressures and random genetic drift in the enzyme sequence (52). To better determine structural features necessary for or unique to activity at low temperatures, a larger database developed with techniques that enable direct comparison of proteins with few structural differences (52) is needed. The availability of the genome of the marine psychrophile C. psychrerythraea strain 34H should enable evaluation of concomitant gene expression and potential interactions between cold-active enzymes and extrinsic factors, such as chaperonins and compatible solutes, as well as the EPS that we included in this study. Further characterization of cold-active enzymes in the presence of such extrinsic factors should provide a better understanding of how these enzymes function in the environment, thus providing insight into the effects of selection pressures on their structure and function.

ACKNOWLEDGMENTS

This research was supported by a Washington State Sea Grant award to J.W.D., by additional support from NSF OPP and LeEsEn awards, by the University of Washington Astrobiology Program, and by an NSF graduate student fellowship to A.L.H.

We thank S. D. Carpenter for technical support, M. W. Adams and J. Holden for advice concerning protein purification techniques, R. Samudrala for 3D modeling and analysis of ColAP, and C. Krems for input in the EPS work.

REFERENCES